Transfections and ELISA assays

All plasmids were linearized with a restriction enzyme prior to introducing them into cells. Cells of the myeloma cell line X63-Ag8.653 were transfected with 12 μg of DNA by electroporation. Cell supernatants were assayed for IgG domains. Briefly, supernatants were incubated in plates coated with anti-human IgG Fc and then bound protein detected using alkaline phosphatase-conjugated anti-human and light chains.

Purification of fusion proteins

Cell supernatants were clarified by centrifugation followed by passage through a 0.45 micron filter. Supernatants were adjusted to 20 mM Tris-HCl, pH 8.3, 150 mM NaCl, and 1 mM EDTA (1 X protein A buffer) and passed over a column of protein A-Sepharose beads. The column was washed in 1X protein A buffer followed by 100 mM Na Citrate, pH 5.0 to elute bound bovine IgG originating from the cell media. Bound fusion protein was then eluted in 100 mM Na Citrate, pH 3.5, neutralized with 0.2 volumes 1 M Tris, and dialyzed against PBS.

TNF cytotoxicity assays

TNF-sensitive WEHI-164 cells (Espevik *et al.*, *J. Immunol. Methods 95*:99-105 (1986)) were plated in 1 μg/ml actinomycin D at 50,000 cells per well in 96-well microtiter plates for 3-4 hours. Cells were exposed to 40 pM TNFα or TNFβ and varying concentrations of fusion protein. The mixture was incubated overnight at 37°C. Cell viability was determined by adding 3-[4,5-dimethyl- thiazol-2-yl]-2, 5diphenyltetrazolium bromide dye (MTT) to a final concentration of 0.5 mg/ml, incubating for 4 hours at 37°C, lysing the cells in 0.1 N HCl, 0.1% SDS and measuring the optical density at 550 nm wavelength.

Saturation binding analyses

Fusion proteins were captured while at a concentration of 10 ng/ml in 96-well microtiter plates coated with goat anti-human Fc antibodies. Varying concentrations of

^{125}I-TNF (34.8 μCi/μg) were added in PBS/1% BSA and allowed to bind for two hours at room temperature. Plates were washed and bound cpm determined. Non-specific binding was determined using an irrelevant antibody.

Several different versions of the p55 fusion proteins were expressed. Unlike what was reported for CD4 (Capon *et al.*, *Nature* 337:525-531 (1989)) and IL-2 (Landolfi, *J. Biol. Chem. 146:*915-919 (1991)) fusion proteins that also included the CHI domain of the heavy chain, inclusion of a light chain proved to be necessary to get secretion of the Ig heavy chain fusion proteins from the murine myeloma cells. The light chain variable region was deleted to enable the TNF R domain on the heavy chain to bind TNF without steric hindrance from the light chain.

The "double fusion" (df) protein, p55-df2, has p55 fused to both the heavy chain and light chain and is therefore tetravalent with regard to p55. p55-sf3 has the p55 receptor (and the same eight amino acids of human J sequence present in p55-sf2 and p55-df2) linked to the hinge region, i.e., the C_H1 domain of the constant region is deleted.

After one or two rounds of subcloning, spent cell supernatant from the various cell lines were yielding 20 μg/ml (for p55-sf2) of fusion protein. The proteins were purified from the spent supernatant by protein A column chromatography and analyzed by SDS-PAGE with or without a reducing agent. Each fusion protein was clearly dimeric in that their M_r estimates from their migration through a non-reducing gel was approximately double the estimated M_r from a reducing gel. However, two bands were seen for p55-sf2 (lane 1) and p55-df2. Two lines of evidence indicated that, in each case, the lower bands did not include a light chain while the upper bands did include a light chain. First, when p55-sf2 containing both bands were passed over an anti-kappa column, the upper band bound to the column (lane 3) while the lower band passed through the column. Second, Western blots have shown that only the upper bands were reactive with anti-kappa antibodies.

It is believed that the versions of these fusion proteins that do not have a light chain (k) were not secreted to a significant degree but rather were primarily released

from dead cells because 1) supernatants from cells transfected with the p55 heavy chain fusion gene and no light chain gene did not have detectable fusion protein until after there was significant cell death, and 2) the ratio of the k- to k+ versions of p55-sf2 increased as cell cultures went from 95% viability to 10% viability.

Example XXVII - p75

To make a p75 heavy chain fusion (p75-sf2), amino acids 1-235 (Smith *et al.*, *Science 248*: 1019-1023 (1990) and Kohno *et al.*, *Proc. Natl. Acad. Sci. 87*:8331-8335 (1990)) were encoded in a fragment prepared using the 5' oligo 5'CACAGCTGCCCGCCCAGGTGGCAT-3' (SEQ ID NO:17) (with the Leu^{1} codon) and the 3' oligo 5'-GTCGCCAGTGCTCCC TT-3' (SEQ ID NO: 18) (with the complement of the Asp^{235} codon). Two other p75 heavy chain fusions (p75P-sf2 and p75P-sf3) were made using the same 5' oligo with the 3' oligo 5'ATCGGACGTGGACGTGCAGA-3' (SEQ ID NO:19). The resulting PCR fragment encoded amino acids 1-182. The PCR fragments were blunt-end ligated into the StuI or EcoRV site of the appropriate vector and checked for the absence of errors by sequencing the inserts completely.

Several different versions of the p75 fusion proteins were also expressed. p75-sf2 has the complete extracellular domain of p75 fused to the heavy chain while p75P-sf2 lacks the C-terminal 53 amino acids of the p75 extracellular domain. p75P-sf3 is the same as p75P-sf2 except that it lacks the C_H1 domain. The region deleted in p75P-sf2 and -sf3 contains sites of O-linked glycosylation and a proline-rich region, neither of which is present in the extracellular domain of p55. Seckinger *et al.*, *Proc. Nat. Acad. Sci. USA 87*:5188-5192 (1990).

Similar to p55-sf2, two bands were seen for p75-sf2 (lane 7) and p75P-sf2 (lane 8).

WEHI cytotoxicity assays

The ability of the various fusion proteins to bind and neutralize human TNFα or TNFβ was tested in a TNF-mediated cell killing assay. Overnight incubation of the murine fibrosarcoma cell line, WEHI 164 (Espevik *et al., J. Immunol. Methods 95*:99-105 (1986)), with 20 pM (1 ng/ml) TNFα results in essentially complete death of the culture. When the fusion proteins were pre-incubated with TNFα (Figure 31A, B and C and Table 1 above) or TNFβ (Figure 32) and the mixture added to cells, each fusion protein demonstrated dose-dependent protection of the cells from TNF cytotoxicity. Comparison of the viability of control cells not exposed to TNF to cells incubated in both TNF and fusion protein showed that the protection was essentially complete at higher concentrations of fusion protein.

Tetravalent p55-df2 showed the greatest affinity for TNFα requiring a concentration of only 55 pM to confer 50% inhibition of 39 pM (2 ng/ml) TNFα (Figure 31A and Table 1). Bivalent p55-sf2 and p75P-sf2 were nearly as efficient, requiring concentrations of 70 pM to half-inhibit TNFα. Approximately two times as much p75-sf2 was required to confer 50% inhibition compared to p55-sf2 at the TNF concentration that was used. The monomeric, non-fusion form of p55 was much less efficient at inhibiting TNFα requiring a 900-fold molar excess over TNFα to inhibit cytotoxicity by 50%. This much-reduced inhibition was also observed with a monomeric, Fab-like p55 fusion protein that was required at a 2000-fold molar excess over TNFα to get 50% inhibition. The order of decreasing inhibitory activity was therefore p55-df2 > p55-sf2 = p75P-sf2 > p75-sf2 >>> monomeric p55.

Surprisingly, the order of decreasing inhibitory activity was different for TNFβ, as presented in Figure 32. p75P-sf2 was most efficient at inhibition requiring a concentration of 31 pM to half-inhibit human TNFβ at 2 pM. Compared to p75P-sf2, three times as much p75-sf2 and three times as much p55-sf2 were necessary to obtain the same degree of inhibition. The order of decreasing inhibitory activity was therefore p75P-sf2 > p75-sf2 = p55-sf2.

Affinity measurements

A comparison was made of the binding affinity of various fusion proteins and TNFα by saturation binding (Figures 33A and 33B) and Scatchard analysis (Figures 33C-33H). A microtiter plate was coated with excess goat anti-Fc polyclonal antibody and incubated with 10 ng/ml of fusion protein in TBST buffer (10 mM Tris-HCl, pH 7.8, 150 NaCl, 0.05% Tween-20) for 1 hour. Varying amounts of ^{125}I labeled TNFα (specific activity - 34.8 μCi/μg) was then incubated with the captured fusion protein in PBS (10 mM Na Phosphate, pH 7.0, 150 mM NaCl) with 1% bovine serum albumin for 2 hours. Unbound TNFα was washed away with four washes in PBS and the cpm bound was quantitated using a y-counter. All samples were analyzed in triplicate. The slope of the lines in (Figures 33C-H) represent the affinity constant, K_a. The dissociation constant (K_d) values (see Table 1) were derived using the equation K_d = 1/K.

Example XXVIII - *In vivo* results

C3H mice were challenged with 5 μg of human TNFα after treatment with an immunoreceptor molecule of the invention. The effect of the treatment was compared with two control treatments. The first control, cA2, is a chimeric mouse/human IgG_1 monoclonal antibody that binds human TNF, and thus is a positive control. The second control, c17-1A, is a chimeric mouse/human IgG_1 irrelevant monoclonal antibody and is thus a negative control. The results of the treatments were as presented in the following Table 19.

TABLE 19

Treatment	Dead Fraction	% Dead
1 μg cA2	5/14	36%
10 μg cA2	1/15	7%
50μg c17-1A	13/15	87%
1 μg p55-sf2	8/15	53%
10 μg p55-sf2	0/15	0%
50 μg p55-sf2	0/15	0%

Mice were injected with 25 μg of p55 fusion protein or a control antibody and 1 hour later were challenged with 1 μg lipopolysaccharide (type J5). Mice were checked 24 hourts later. The results are presented in the following Table 20.

TABLE 20

Treatment	Dead Fraction	% Dead
Control Antibody	11/11	100%
p55-sf2	0/13	0%

All references cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional methods steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

EQUIVALENTS

Those skilled in the art will know, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.